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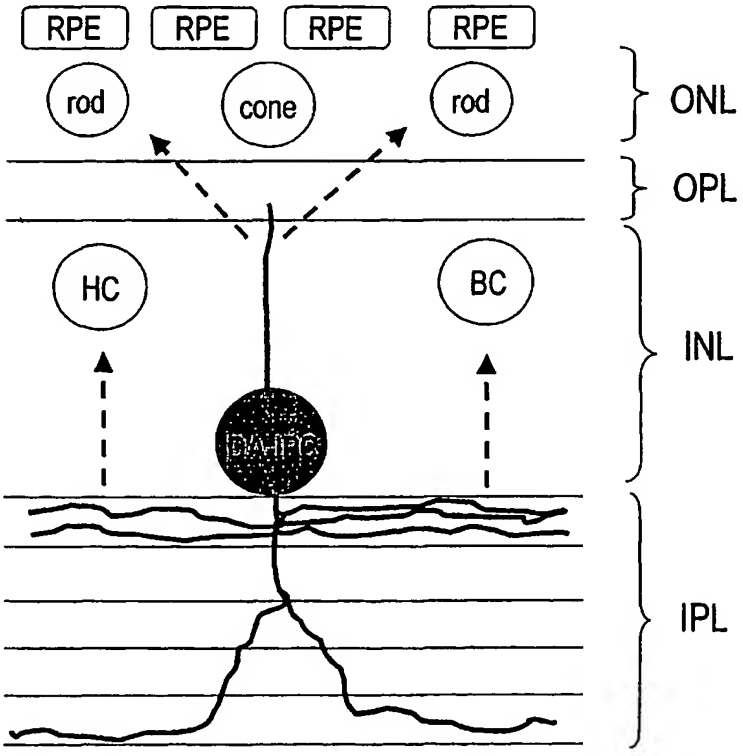

(57) Abstract: A method of enhancing photoreceptor survival in organ culture, cell culture and *in vivo* using dopamine receptor antagonists and agents that deplete dopamine is described.

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COMPOSITION AND METHODS FOR TREATING PHOTORECEPTOR DEGENERATION

This application claims the benefit of priority to U.S. provisional application No. 60/201,302, filed May 2, 2000.

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates generally to the prevention of photoreceptor degeneration in organ cultures, cell cultures and *in vivo*.

2. Description of the Related Art

Photoreceptors are a specialized subset of retinal neurons consisting of rods and cones. A number of diseases of the retina involve the progressive degeneration and eventual death of photoreceptors. Such diseases include retinitis pigmentosa, age-related macular degeneration and other maculopathies or retinal detachment. Some work has been done in using various trophic factors to rescue photoreceptors from death. For instance, photoreceptors can be rescued by basic fibroblast growth factor (bFGF) in Royal College of Surgeons (RCS) rats and in albino rats that have been damaged by exposure to constant light. Faktorovich *et al.*, *Nature*, 347: 83-86 (1990). RCS rats have an inherited mutation of the gene expressed in the retinal pigment epithelium (RPE) that results in the failure of the RPE to phagocytize the continuously shed portions of the photoreceptor outer segments and causes photoreceptor degeneration and eventually cell death. A single injection of bFGF into the vitreous body or into the subretinal space, the extracellular space surrounding rods and cones, at the onset of the degeneration transiently rescues photoreceptors. Faktorovich *et al. supra*. Similarly, bFGF injected into the subretinal space of a light damaged model of albino rats, protects photoreceptors from the effects of light damage and prevents cell death. LaVail *et al., PNAS, USA* 89: 11249-11253 (1992). Other trophic factors studied for this purpose

are acidic fibroblast growth factor (aFGF), brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), interleukin-1 beta, neurotrophin-3 (NT-3), insulin-like growth factor II (IGF-II), tumor necrosis factor-alpha (TNF-alpha), platelet derived growth factor (PDGF) and glial derived neurotrophic facotor (GDNF). LaVail *et al, supra.*

Although it has previously been shown that the growth factors ciliary neurotrophic factor (CNTF) and brain-derived neurotrophic factor (BDNF) in combination can block photoreceptor degeneration in a 4 week organ culture model of the *rd* retina (Ogilvie *et al., Exp. Neurol.* 161: 676-685 (2000), evidence suggests that these factors do not have a direct action on photoreceptors. Koide *et al., Neurosci Lett* 185: 183-186 (1995); Perez and Caminos, *Neurosci. Lett* 183: 96-99 (1995); Rickman and Brecha, *Vis Neurosci* 12: 215-222 (1995); Ugolini *et al., Brain Res.* 704: 121-124 (1995); Kirsch *et al., J. Neurochem* 68: 979-990 (1997); Llamosas *et al., Anat Embryol* 195: 337-344 (1997). They may act indirectly through support cells such as retinal pigment epithelial or Muller cells. Harada *et al.,Neuron* 26: 533-541 (2000); Ogilvie *et al., supra.* Alternatively, growth factors could act through dopaminergic neurons.

Since the BDNF receptor has been localized to dopaminergic neurons in the retina, (Cellerino and Kohler, *J. Comp. Neurol.* 386: 149-160 (1997), the inventors considered that the growth factors might act through dopaminergic neurons to increase photoreceptor cell survival. To test this possibility, the inventors proposed to add both CNTF and BDNF in the presence of dopamine antagonists. Initial control experiments involved growing *rd* retinas in organ culture in the presence of D_1 or D_2 family receptor antagonists in order to determine any paracrine effects of dopamine on photoreceptors in this system. Surprisingly, the inventors discovered that, in the absence of growth factors, dopamine antagonists prevent photoreceptor degeneration for the duration of the 4 week *in vitro* paradigm. Conversely, the inventors found that dopamine is necessary for photoreceptor degeneration. Thus, the present invention is based on the discovery that antagonists of dopamine receptors prevent photoreceptor degeneration.

SUMMARY OF THE INVENTION

In one embodiment, the invention relates to a method of enhancing photoreceptor survival in retina organ culture comprising contacting said photoreceptors in culture with a compound that is a dopamine receptor antagonist or is an agent that depletes dopamine.

In another embodiment, the invention relates to a retina organ culture comprising retinal tissue and a dopamine receptor antagonist or an agent that depletes dopamine.

In another embodiment, the invention relates to a method of treating photoreceptor degeneration comprising administering to the retina of a subject in need thereof a therapeutically effective amount of an antagonist of dopamine receptors in photoreceptor cells or an agent that depletes dopamine.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic of the retina.

Figure 2 is a schematic of a method of culturing the retina.

Figure 3 shows that TH-positive neurons are present in antagonist treated organ cultures.

Figure 4 shows the effects of dopamine antagonists on photoreceptor cell survival in *rd* retinal organ cultures. Retinas from wild type (A) and *rd* (B,C,D) mice were harvested at postnatal day 2 and grown in organ culture for 27 DIV. Wild type retinas maintained approximately 5 rows of cells in the ONL (A) while the ONL of untreated *rd* cultures was reduced to a monolayer (B). No photoreceptor degeneration was seen in *rd* organ cultures treated with 100 nM sulpiride, a D_2 family antagonist (C). SCH-23390 (20 nM), a D_1 family antagonist also increases photoreceptor survival in *rd* organ cultures (D). Quantitative analysis of photoreceptor survival as measured by ONL thickness is shown in (E). RPE, retinal pigment epithelium; IS/OS, photoreceptor inner and outer segments; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer. Bar, 15 μm. * $p < 0.05$ vs. wild type; † $p << 0.001$ vs. *rd*, student's t-test. Error bars

indicate standard error. The number of cultures is indicated on the column for each condition.

Figure 5 shows the effects of dopamine depletion on photoreceptor cell survival in *rd* retinal organ cultures. Retinas were grown in organ culture for 27 DIV with either control media or 6-hydroxydopamine (6-OHDA) and pargyline added to the media on the first 2 days *in vitro* and again after 1 week. 6-OHDA treated *rd* organ cultures showed no degeneration (A) and could not be distinguished from wild type organ cultures (see figure 4B). When 20 μM of the dopamine agonist ADTN was added to 6-OHDA treated organ cultures, photoreceptor degeneration was comparable to untreated *rd* organ cultures (B). Bar, 15 μm. † $p < 0.001$ vs. *rd*; ** $p < 0.001$ vs. wild type; student's t-test. $N = 4$ for each condition. Error bars indicate standard error.

Figure 6 shows the effects of dopamine inhibition on opsin expression in *rd* retinal organ cultures. Opsin immunohistochemistry was performed on untreated wild type (A) and *rd* (B) organ cultures as well as *rd* organ cultures treated with sulpiride (C), SCH-23390 (D), or 6-OHDA (E). In all cases, intense opsin immunoreactivity is seen in the outer nuclear layer and in residual inner and outer segments. Neither dopamine inhibition nor dopamine depletion alters opsin expression. IS/OS, photoreceptor inner and outer segments; ONL, outer nuclear layer. Bar, 15 μm.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides methods and compositions for the treatment of photoreceptor degeneration. The invention is based on the discovery that dopamine is necessary for photoreceptor degeneration and that dopamine antagonists or the elimination of dopamine prevents photoreceptor degeneration.

Dopamine is an endogenous neurotransmitter in the retina that affects most, if not all, cell types in the vertebrate retina, playing a role in the transition from scotopic to photopic vision. Dopaminergic neurons in the mouse retina are interplexiform cells with extensive processes on the outer margin of the inner plexiform layer (IPL), sparser processes

throughout the IPL, and an ascending process extending to the outer plexiform layer (OPL). Witkovsky and Schuette *Vis. Neurosci.* 7: 113-124 (1991). D_1 family dopamine receptors, characterized by activation of adenelyl cyclase, are found in both the OPL and IPL, primarily on horizontal and bipolar cells, respectively. D_2 family dopamine receptors, which inhibit adenelyl cyclase, are also found in both plexiform layers. Additionally, D_4 receptors are located on the inner segments of photoreceptors where dopamine has been shown to act in a paracrine fashion. Cohen *et al. PNAS USA* 89: 12093-12097 (1992).

Thus, in one embodiment, the invention relates to a method of enhancing photoreceptor survival in a retina organ culture. By "enhancing" is meant a statistically significant increase in the number of surviving photoreceptors in the organ culture compared to the untreated *rd* organ culture (i.e. control media) as measured by counting the number of cells, indicating the thickness of the ONL or the density of cells in the ONL. Complete inhibition of degeneration is indicated if the counts in treated *rd* organ cultures is not significantly different from untreated wild type organ cultures. By "retina organ culture" is meant a retina organ culture as described in Ogilvie *et al., Neurosci Meth.* 87: 57-65 (1999), which is herein incorporated by reference. Example 1 presents a protocol for preparing and maintaining a retina organ culture. However, the skilled artisan would understand that this term includes other types of retina organ cultures. Other illlustrative examples are described in Caffé, A.R. and Sanyal, S. (1991) Retinal degeneration in vitro: Comparison of postnatal retinal development of normal, rd and rds mutant mice in organ culture. In R.E. Anderson, J.G. Hollyfield and M.M. LaVail Eds., *Retinal Degenerations*, CRC Press, Boca Raton, FL, pp. 29-38; Feigenspan, A., Bormann, J. and Wässle, H. (1993) Organotypic slice culture of the mammalian retina, *Vis. Neurosci.*, 10: 203-217; Hild, W. and Callas, G. (1967) The behavior of retinal tissue in vitro, light and electron microscopic observations, *Z. Zellforsch.*, 80: 1-21; Lucas, D.R. (1958) Inherited retinal dystrophy in the mouse: its appearance in eyes and retinae cultured in vitro, *J. Embryol. exp. Morph.*, 6: 589-592.
Sheedlo, H.J. and Turner, J.E. (1995) Photoreceptor survival and development in culture, *Prog. Ret. Eye Res.*, 15: 127-137; Sidman, R.L. (1961) Tissue culture studies of inherited retinal dystrophy, *Dis. Nerv. Sys.*, 22: 14-20; Sparrow, J.R., Hicks, D. and Barnstable, C.J. (1990) Cell commitment and differentiation in explants of embryonic rat neural retina. Comparison with the developmental potential of dissociated retina, *Dev. Brain Res.*, 51: 69-84; Tamai, M., Takahashi, J., Noji, T. and Mizuno, K. (1978) Development of

photoreceptor cells in vitro: Influence and phagocytic activity of homo- and heterogenic pigment epithelium, *Exp. Eye Res.*, 26: 581-590; Tansley, K. (1933) The formation of rosettes in the rat retina, *Brit. J. Ophthalmol.*, 17: 321-336; and Trowell, O.A. (1954) A modified technique for organ culture in vitro, *Exp. Cell Res.*, 6: 246-248.

The survival of the photoreceptors is enhanced by administration to the organ culture of a dopamine receptor antagonist or an agent that depletes dopamine. The term "antagonist" refers to an agent which directly or indirectly combines with receptors on a cell and inhibits the stimulation of a response. Within the scope of this definition are small molecules which bind dopamine receptors in photoreceptor cells, antibodies against such receptors and chemical compounds. Examples of dopamine antagonists include, but are not limited to AJ76, Alprenolol, Amisulpride, Bromocryptine, (+) Butaclamol, (-) Butaclamol, Chlorpromazine, cis-flupenthixol, cis-piflutixol, Clozapine, Dihydroergocryptine, Domperidone, Eticlopride, Fluphenazine-n-mustard (FNM), Haloperidol, IBZM, Iodosulpride, Ketanserin, Metoclopramide, Olanzapine, Pimozide, PNU-99194A, PNU-101387, Prazosin, Prochlorperazine, Raclopride, SCH23388, SCH23390, SCH23982, SCH39166, Spiperone, Sulpiride, Thioproperazine, Thioridazine, UH232, and Yohimbine . In a preferred embodiment, the antagonist of the invention is an antagonist of the D_1 or D_2 receptor families. In one preferred embodiment, such antagonist is sulpiride; in another it is SCH-23390. *See* Sokoloff *et al., Nature* 347: 146-151 (1990), Van Tol. *et al.Nature* 350: 610-614 (1991) and Vallone *et al. Neurosci. and Biobehavioral Reviews* 24: 125-132 (2000), all of which are herein incorporated by reference.

An "agent that depletes dopamine" includes any such agent known to deplete dopamin, such as 6-hydroxydopamine (6-OHDA), a toxin specific for dopaminergic neurons, which leaves the receptors intact, but depletes the level of dopamine so that the receptors are not activated.

In another embodiment, the invention relates to a method of screening for antagonists of dopamine receptors in photoreceptor cells comprising comparing the extent of degeneration of photoreceptors in two groups of *rd* mouse retina organ tissue, the first group comprising *rd* mouse retina organ tissue that has been contacted with the subject compound and the second group comprising *rd* mouse retina organ tissue that has not been contacted

with the subject compound, wherein a decrease in the extent of degeneration indicates that the subject compound is an antagonist of dopamine receptors in the retina.

Other antagonists within the scope of the invention can be determined using the above assay. Of course, the skilled artisan would know of other animal models suitable for use in this assay. Such animal models include the RCS rat and albino rat described above, as well as *rd2, rd3, rd4, rd5* and various transgenic mice with retinal degeneration. *See* Faktorovich *et al., Nature*, 347: 83-86 (1990). However, in the preferred embodiment, the animal model is the *rd* mouse. The *rd* mouse is a well characterized animal model of photoreceptor degeneration that carries an autosomal recessive defect in the ß-subunit of the rod specific cGMP-PDE gene. This defect results in loss of rod photoreceptors with the first three postnatal weeks followed by a slow degeneration of the cones. *See* Chang *et al. Neuron* 11: 595-605 (1993); Lolley *et al., Invest. Ophthalmol. Vis Sci* 35: 358-362 (1994); and Portera-Cailliau e*t al. PNAS USA* 91: 974-978 (1994).

In yet another embodiment, the above assay could be performed using cultured cells that express a dopamine receptor. Such cells could be a mammalian cell line transfected with DNA encoding a dopamine receptor. *See* U.S. Patent No. 6,214,615, which is herein incorporated by reference.

In one embodiment, the dopamine antagonist of the invention is an antibody against a dopamine receptor. "Antibody" refers to a polypeptide comprising a framework region encoded by an immunoglobulin gene or fragments thereof that binds and recognizes an antigen. The recognized immunoglobulin genes include the kappa, lambda, alpha, gamma, delta, epsilon, and mu constant region genes, as well as the myriad immunoglobulin variable region genes. Light chains are classified as either kappa or lambda. Heavy chains are classified as gamma, mu, alpha, delta, or epsilon, which in turn define the immunoglobulin classes, IgG, IgM, IgA, IgD and IgE, respectively. An exemplary immunoglobulin (antibody) structural unit comprises a tetramer. Each tetramer is composed of two identical pairs of polypeptide chains, each pair having one "light" (about 2 kD) and one "heavy" chain (about 0-70 kD). Antibodies exist, e.g., as intact immunoglobulins or as a number of well characterized fragments produced by digestion with various peptidases. While various antibody fragments are defined in terms of the digestion of an intact antibody, one skilled in the art will appreciate that such fragments may be synthesized *de novo* chemically or via recombinant DNA methodologies. Thus, the term antibody, as used herein,

also includes antibody fragments produced by the modification of whole antibodies, those synthesized *de novo* using recombinant DNA methodologies *(e.g.,* single chain Fv), humanized antibodies and those identified using phage display libraries *(see, e.g.,* Knappik *et al. J. Mol. Biol.* 296: 57-86 (2000), McCafferty *et al., Nature* 348:2-4 (1990)), for example. For preparation of antibodies – recombinant, monoclonal, or polyclonal antibodies – any technique known in the art can be used in this invention *(see, e.g.,* Kohler & Milstein, *Nature* 26:49-497 (1987); Kozbor *et al., Immunology Today 4:* 72 (1983); Cole *et al.,*pp. 77-96 in *Monoclonal Antibodies and Cancer Therapy,* Alan R. Liss, Inc. (1989).

Techniques for the production of single chain antibodies (*See* U.S. Patent 4,946,778) can be adapted to produce antibodies to polypeptides of this invention. Transgenic mice, or other organisms such as other mammals, may be used to express humanized antibodies. Phage display technology can also be used to identify antibodies and heteromeric Fab fragments that bind to selected antigens. S*ee, e.g.,* McCafferty *et al., Nature* 348:2-4 (1990); Marks *et al., Biotechnology* :779-783 (1992).

An "anti-dopamine receptor" antibody is an antibody or antibody fragment that binds a polypeptide encoded by a dopamine receptor gene, cDNA, or a subsequence thereof.

In another embodiment, the antagonist of the invention is an antisense molecule comprising the antisense of DNA encoding a dopamine receptor.

In another embodiment, the invention relates to a method of treating retinal cell degeneration comprising administering to the retina of a patient in need thereof a therapeutically effective amount of an antagonist of dopamine receptors in photoreceptor cells or any agent that depletes dopamine, as described above. The antagonist or agent may be administered intraocularly, *i.e.* via injection through the vitreous or subretinal space, locally by insertion into the tissue surrounding the eye, systemically through an oral route or by subcutaneous, intravenous or intramuscular injection or via catheter or implant. The antagonist or agent of the invention may be admininstered prior to the onset of a retina degenerative condition, to prevent its occurrence such as during eye surgery, or for the benefit of persons susceptible to a degenerative disease, immediately after the onset of a pathological condition or during the occurrence of an acute or protracted condition. Thus, by "treating," is meant preventing of the onset of degeneration or lessening, ameliorating, curing or at least partially arresting symptoms and/or complications. Amounts effective for this will depend on, *e.g.*, the antagonist or agent composition, the manner of administration, the stage

and severity of the disease being treated, the weight and general state of health of the patient, and the judgment of the prescribing physician. In some embodiments of the invention, an effective dose is an amount of the antagonist that is sufficient to inhibit mRNA transcription of the dopamine receptor.

The antagonist or agent of the invention may be formulated in a pharmaceutical composition. For instance, in one embodiment, the antagonist is admixed with a carrier or is suspended in a solution. The compositions may further contain pharmaceutically acceptable auxilliary substances as required to approximate physiological conditions such as pH adjusting, and buffering agents, tonicity adjusting agents, wetting agents and the like. *See Remington's Pharmaceutical Sciences,* 18th Ed. (1990, Mack Publishing Co., Easton, PA 18042, pages 1435-1712 of which is hereby incorporated by reference. Additionally, the antagonist or agent of the invention may be administered via liposomes. Liposomes include emulsions, foams, micelles, insoluble monolayers, liquid crystals, phospholipid dispersions, lamellar layers and the like.

In another embodiment, the retinal cells of the patient may be treated with the antagonist or agent of the invention *ex vivo* and then transplanted back into the retina of the patient. For instance, the retinal cells may be transfected with a vector expressing DNA encoding the antagonist of the invention. Photoreceptor cell transplantation studies designed to replace defective or lost cells due to retinal disease or damage have been performed successfully in animal models of retinal degeneration. *See* Silverman and Hughes, *Invest. Ophthalmol. Vis. Sci.*, 30: 1684-1690 (1989); Gouras *et al., Neuroophthalmol.* 10: 165-176 (1990). In another embodiment, the invention contemplates that photoreceptor cells may be obtained from donor eyes and maintained in culture as described herein. The cells would then be used as a source of purified photoreceptors to be transplanted via the subretinal space into the retina of patients suffering from retinal disease or damage. These patients will be treated with immunosuppressive therapies to eliminate immunological responses and rejection of the grafted cells. The *ex vivo* donor retinas will be cultured in the presence of the antagonist or agent of the invention and perhaps other trophic factors, as described above, in order to enhance their growth and survival. Such growth factors could include **CNTF, BDNF, BFGF, or GDNF.** The patients that receive photoreceptor cell transplants will be treated with intravitreal injections of the antagonist that will be needed to promote the survival and the maturation of the grafted photoreceptors.

In another embodiment, the invention relates to gene therapy, i.e. delivery of DNA that encodes an antagonist of the invention to target cells in the retina. For example, in one embodiment a nucleic acid construct containing such DNA may be containined in an adeno-associate virus vector or other appropriate delivery vector. Hefti, *J. Neurobiol.*, 25: 1418-1435 (1994). Alternative viral vectors include, but are not limited to, retrovirus, herpes simplex virus and papilloma virus vectors. Physical transfer, either *in vivo* or *ex vivo* as appropriate, may also be achieved by liposome-mediated transfer, direct injection (naked DNA), electroportation, calcium phosphate precipitation or micropartical bombardment (gene gun), or other methods well known to the skilled artisan.

In yet another embodiment, the invention relates to a retina organ culture comprising retinal tissue and a dopamine receptor antagonist or dopamine depleting agent. An exemplitive culture is described in Example 1, below. In one embodiment, the dopamine receptor antagonist is an antagonist of a member of the D_1 or D_2 family of dopamine receptors. The retina organ culture according to one embodiment is human retinal tissue, in another embodiment it is *rd* mouse tissue. Preferrably, the antagonist is supiride or SCH-23390 (RBI Signaling, Natick, MA). The organ culture of the invention also may include trophic factors, such as ciliary neurotrophic factor (CNTF), brain-derived neurotrophic factor (BDNF), glia-derivied neurotrophic factor (GDNF), basic fibroblast growth factor (bFGF). Preferrably, the dopamine depleting agent according to this embodiment is 6-OHDA.

The following examples exemplify the invention but are not intended to limit the scope of the invention in any way. All of the above citations and following citations are herein incorporated by reference.

Example 1: Protocol for Establishing Neonatal Mouse Isolated Retina Cultures

Use pigmented *rd* mice pups.

1. On the bench top, anesthetize each pup by chilling on ice, and then sacrifice by decapitation.
2. Dip each head into 70% ETOH.
3. On the clean bench (for this and all future steps) enucleate each eye by doing a blunt dissection using #4 forceps.
4. Drop each globe into cold DMEM (Gibco#11965)* plus Fungizone* (2X=2.5ug/ml) in a 35mm petrie dish.
5. 'Clean up' (using fine forceps) each globe of extraneous connective tissue.

6. Place the globes into a 0.5% Proteinase K (Boehringer Mannheim #745 723)*solution (in DMEM) in a fresh petrie dish and incubate for 7 minutes at 37°C.
7. At room temperature, rinse the tissue in DMEM plus 10% FCS(fetal calf serum*) Fungizone* (1.25ug/ml) and then into DMEM plus Fungizone (1.25ug/ml) but this time without serum.
8. Using two pair of #5 forceps and starting at the limbus border, carefully peel away the sclera, and choroid leaving the RPE attached to the retina. Next remove the anterior segment (cornea, lens and vitreous).
9. Gently scooping the tissue up on the tip edge of a pair of forceps move the retinas with RPE to a fresh petrie dish containing DMEM plus 10% FCS plus Fungizone (1.25ug/ml). Incubate the tissue for approximately 30 minutes at 37°C to detach the RPE.
10. The RPE (which is now mostly rolled up and separated from the retina) can be gently teased away from the retina using fine forceps. At this point the RPE can be collected, dissociated and cultured or discarded if not needed.
11. Using a disposable transfer pipette in which the end has been cut to enlarge the opening, individually move each retina together with a few drops of media onto a Millipore Millicell- CM** (30mm)cell culture insert. Place one retina/insert and position photoreceptor side down. Suck off enough media to prevent the retina from floating around while attempting to tease it out flat (but not so much that the tissue sticks down prematurely and can not be manipulated). To tease and flatten use a fine tip glass probe made from a pulled and then closed bore tip (fire polished) Pasteur pipette. For 6 well/plate size plates each insert is situated over 1.0 mls of media. Remove any excess media surrounding the retina leaving only a light film to cover it.
12. Media for control tissue is DMEM plus 10% FCS plus Fungizone (1.25 ug/ml). Dopamine antagonists or dopamine depleting drugs are added to the media immediately prior to use.
13. Culture the retinas in the incubator at 37°C and with 5% CO2. Feed the cultures daily replacing the media in the entire dish with freshly prepared media and drug each time.

Example 2: Retinal Organ Culture Studies

A. METHODS

Organ cultures. Retinal organ culture procedures have been described previously in detail (Ogilvie *et al.*, J. Neurosci. Meth. 87: 57-65 (1999)). Retinas were isolated from retinal dystrophic (*rd*) or from wild type control mice on a C57BL/6 background at postnatal day 2. Animals were handled in accordance with institutional guidelines and the Society for Neuroscience Policy on the Use of Animals in Neuroscience Research. After separation from the retinal pigment epithelium, the isolated retina was placed photoreceptor side down on a Millicell-CM culture insert (Millipore, Bedford, MA) with media maintained at the level of the membrane interface. The cultures were maintained at 37°C, 5% CO_2 for 27 days. Control media was comprised of Dulbecco's Modified Eagle's Media (DMEM, Gibco #11965, Rockville, MD) with 10% fetal calf serum (FCS, Summit Biotechnology, Ft. Collins, CO) and 1.25 μg/ml Fungizone (Sigma, St. Louis, MO).

For dopamine depletion studies, 100 μM each of 6-hydroxydopamine (6-OHDA, Sigma, St. Louis, MO) and pargyline (Sigma, St. Louis, MO) were added to the media on the first two days and repeated with 50 μM each on days 7 and 8. The drugs were administered in two pulses since immature neurons are less sensitive to 6-OHDA. For the remaining days *in vitro* (DIV), organ cultures were fed with control media every 2-3 days for 27 days. In some experiments, 2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene (ADTN, RBI Signaling, Natick, MA) was also added to the media, which was replaced daily. For dopamine receptor studies, antagonists for either D_1 (20 nM SCH-23390) or D_2 (100 nM sulpiride) dopamine receptors (RBI Signaling, Natick, MA), were added to the media immediately prior to feeding; cultures were fed daily.

Histology. After 27 days *in vitro* (27 DIV), organ cultures for quantitative analysis were fixed in 2.5% glutaraldehyde and 2% paraformaldehyde overnight. Tissue was postfixed in 1% osmium tetroxide followed by 1% uranyl acetate, rinsed, dehydrated and embedded in Epon-Araldite for histological evaluation of 1 μm sections. For immunohistochemistry, organ cultures were fixed in 4% paraformaldehyde for 1 hr, rinsed, and cryoprotected in 30% sucrose overnight. The tissue was frozen in O.C.T. (Sakura, Torrance, CA), cut into 8-10 μm cryostat sections, and stored at –80°C.

Immunocytochemistry. Slides were thawed, rinsed in phosphate buffered saline (PBS), and blocked in 2% normal goat serum (NGS, Sigma, St. Louis, MO)/PBS. Tissue was incubated overnight with 4D2 anti-rhodopsin (graciously provided by David Hicks, INSERM, Strasbourg) diluted 1:1000 in PBS containing 0.3% Triton X-100 and 2% NGS at 4°C. Tissue was washed, blocked in 2% NGS, and incubated in goat anti-mouse IgG, Cy3-conjugated (Amersham, Piscataway, NJ) at a dilution of 1:500 for 1 hour at room temperature. Slides were washed in PBS and coverslipped with aqueous mounting media.

Data Analysis. Analysis of photoreceptor survival was performed on 1 μm plastic sections by a blinded observer as previously described (Ogilvie *et al.*, 1999, *supra*). Briefly, the center point of each retinal section was determined and two regions, 100 μm on either side of that point, were randomly selected for quantitative analysis. The thickness of the outer nuclear layer (ONL) was determined by averaging 5 counts from each region (20 μm apart). Each count determined the number of ONL cells in a vertical column touching a single grid line on a reticule. The total number of ONL nuclei were counted in both regions to determine the density of cells in the ONL. In all cases, density corresponded to the thickness of the ONL. Statistical significance was determined using a student t-test.

B. RESULTS

Dopamine receptor antagonists increase photoreceptor survival

To determine whether dopamine plays a role in photoreceptor degeneration in the *rd* mouse retina, 100 nM sulpiride, a D_2 family antagonist, was added to retinal organ cultures for 27 DIV. In untreated *rd* retinal organ cultures, only a monolayer of cells survive in the ONL after this period. Normal retinas maintain a thickness of 4.9 cells (± 0.3) in the ONL, thinner than *in vivo* as a result of tissue spreading in the culture dish. *rd* organ cultures treated with 100 nM sulpiride maintained an average of 4.4 cells (± 0.13) in the ONL (Fig. 1). The thickness and cell density in the ONL of sulpiride-treated *rd* organ cultures were not significantly different from those of untreated normal retinal organ cultures.

The effects of 20 nM SCH-23390, a D_1 receptor antagonist, were tested in retinal organ cultures. Similar to results seen with sulpiride, the addition of SCH-23390 resulted in a dramatic increase in photoreceptor survival, as indicated by ONL thickness (3.7 ± 0.1 cells),

and density, although photoreceptor survival was slightly less than seen in normal retinas ($p < 0.05$; Figure 4).

Depletion of dopamine increases photoreceptor survival

In order to determine whether the dopamine receptor antagonists might be acting in a nonspecific fashion on photoreceptor survival, we depleted retinal dopamine by treatment of rd retinal organ cultures with the specific dopaminergic toxin, 6-OHDA and pargyline as described in the Methods. No TH-positive neurons could be identified in 6-OHDA-treated organ cultures, consistent with the elimination of dopaminergic neurons (data not shown). Dopamine depletion resulted in rd retinal organ cultures that could not be distinguished from normal C57 retinal organ cultures (Fig. 5). The thickness (4.6 ± 0.32 cells) and density of the ONL were completely preserved. Inner and outer segment material in dopamine depleted cultures was comparable to that of normal retinal cultures and greater than that seen in cultures treated with dopamine antagonists. The addition of 20 μM ADTN, a dopamine agonist, to organ cultures treated with 6-OHDA resulted in photoreceptor degeneration comparable to that seen in untreated rd retinal cultures (Fig. 5B).

Dopamine antagonists do not block terminal differentiation of opsin expression in photoreceptors

The rapid photoreceptor degeneration in the *rd* mouse retina makes this a good model for organ cultures that can be maintained for a limited time. However, the early and rapid effects of the *rd* mutation result in pathology that begins as early as the first postnatal week, before photoreceptor differentiation is complete (Sanyal and Bal, *Z.Anat. Entwickl-Gesch.* 142: 219-238 (1973); Farber *et al., Prog. Ret. Res.* 13: 31-64 (1994). This makes it possible to increase survival of photoreceptors by blocking terminal differentiation of the phototransduction pathway. Immunohistochemistry was used to examine opsin expression in *rd* retinal organ cultures treated with dopamine antagonists or 6-OHDA. Opsin labeling was found throughout the ONL in cultures treated with sulpiride, SCH-23390, or 6-OHDA comparable to that seen in C57 control cultures (Fig. 6). Intense labeling was seen in residual inner and outer segments of cultures treated with either antagonist or with dopamine depletion and in the inner and outer segment material of untreated control cultures. These

results indicate that neither dopamine inhibition nor dopamine depletion alters photoreceptor terminal differentiation, as indicated by opsin expression.

C. DISCUSSION

It has been demonstrated that photoreceptor degeneration can be blocked by inhibition of dopamine in the *rd* mouse retina grown in organ culture. This result is achieved with either D_1 or D_2 family receptor antagonists or through dopamine depletion with the specific dopaminergic neurotoxin, 6-OHDA. In the latter case, the readdition of the specific dopamine agonist, ADTN, induced photoreceptor degeneration, thus demonstrating that depletion of dopamine was the basis of the protection afforded by 6-OHDA.

WHAT IS CLAIMED IS:

1. A method of enhancing photoreceptor survival in retina organ culture comprising contacting said photoreceptors in culture with a compound that is a dopamine receptor antagonist.

2. The method of claim 1, wherein the dopamine receptor antagonist is an antagonist of a member of the D_1 or D_2 receptor family.

3. The method of claim 1, wherein the antagonist is selected from the group consisting of SCH-23390 and sulpiride.

4. A retina organ culture comprising retinal tissue and a dopamine receptor antagonist.

5. The retina organ culture of claim 4, wherein the dopamine receptor antagonist is an antagonist of a member of the D_1 or D_2 receptor family.

6. The retinal organ culture of claim 5, wherein the antagonist is selected from the group consisting of SCH-23390 and sulpiride.

7. The retina organ culture of claim 4, wherein the retina tissue is human retina tissue.

8. The retina organ culture of claim 4, wherein the retina tissue is from an *rd* mouse.

9. The retina organ culture of claim 4, further comprising a growth factor selected from the group consisting of ciliary neurotrophic factor (CNTF), brain-derived neurotrophic factor (BDNF), glia-derivied neurotrophic factor (GDNF), and basic fibroblast growth factor (bFGF).

10. A method of treating photoreceptor degeneration comprising administering to the retina of a subject in need thereof a therapeutically effective amount of an antagonist of dopamine receptors in photoreceptor cells.

11. The method of claim 10, wherein the dopamine receptor antagonist is an antagonist of a member of the D_1 or D_2 receptor family.

12. The method of claim 11, wherein the antagonist is selected from the group consisting of SCH-23390 and sulpiride.

13. A method of enhancing photoreceptor survival in retina organ culture comprising contacting said photoreceptors in culture with a compound that is an agent that depletes dopamine.

14. The method of claim 13, wherein said agent is 6-OHDA.

15. A retina organ culture comprising retinal tissue and an agent that depletes dopamine.

16. The retina organ culture of claim 15, wherein said agent is 6-OHDA.

17. A method of treating photoreceptor degeneration comprising administering to the retina of a subject in need thereof a therapeutically effective amount of an agent that depletes dopamine.

18. The method of claim 17, wherein said agent is 6-OHDA.

Fig. 1

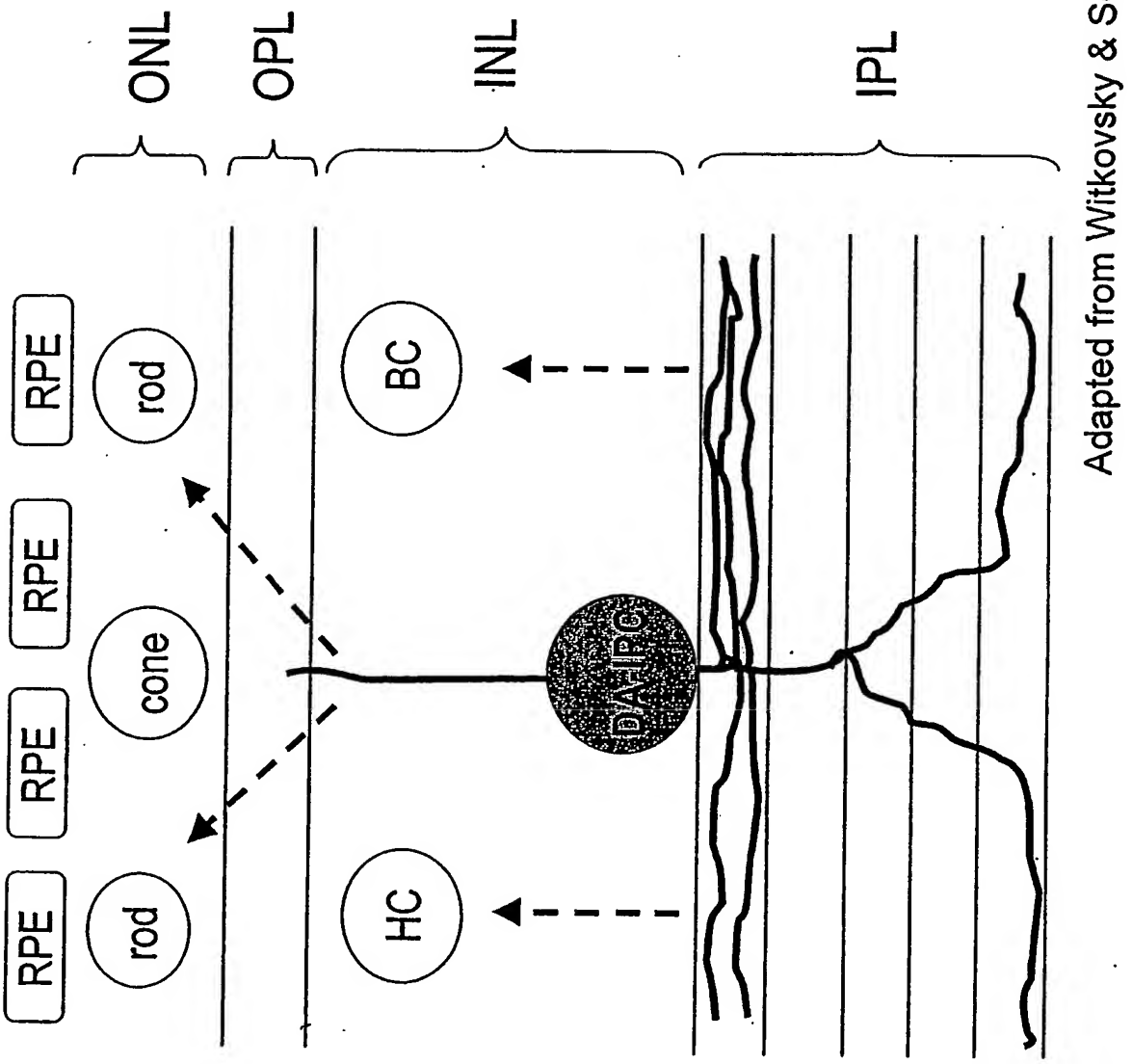
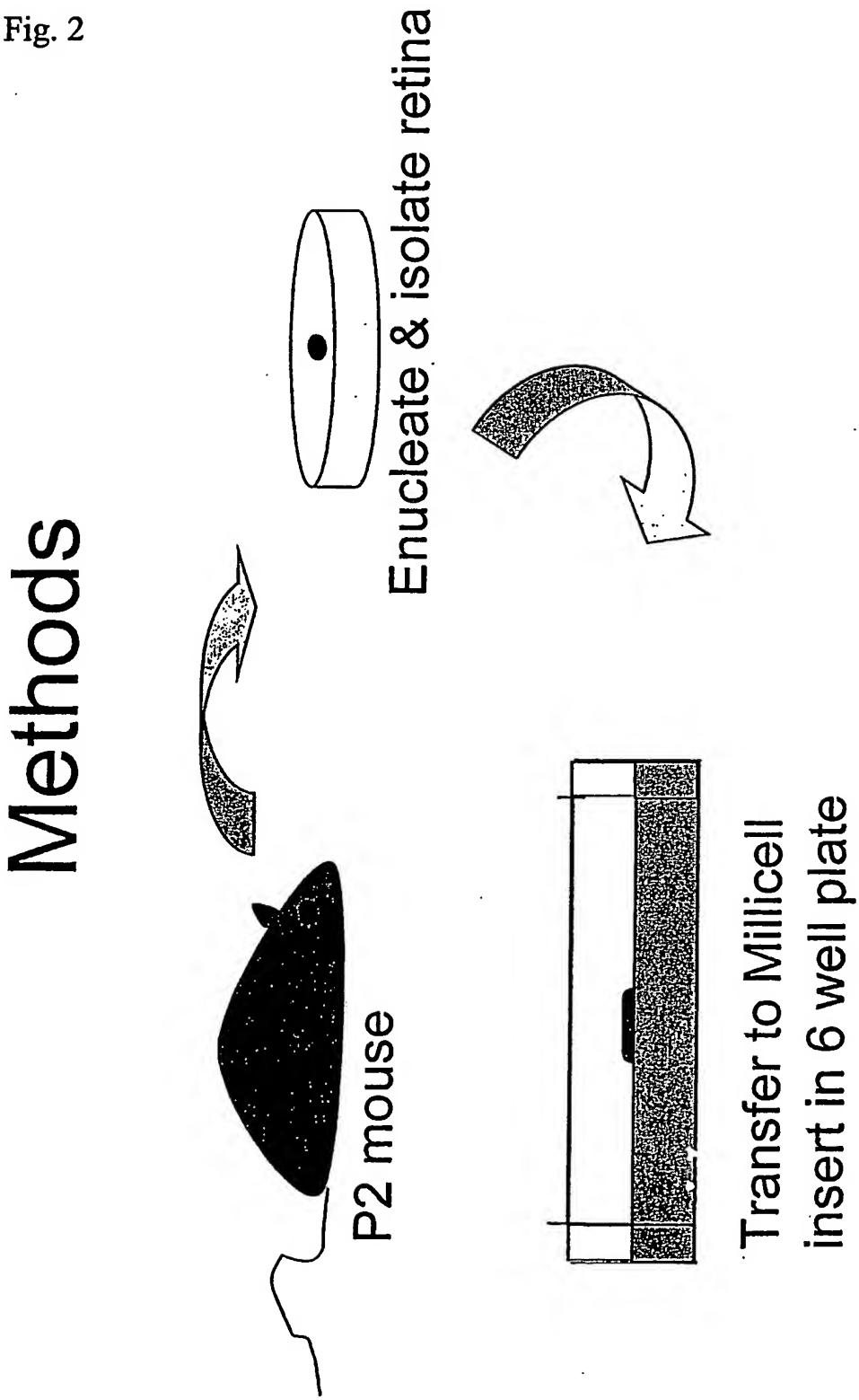

ONL
OPL
INL
IPL
RPE
RPE
RPE
RPE
rod
cone
rod
BC
HC
DA-IPG
Adapted from Witkovsky & Schutte, 1991

Fig. 2


Methods
P2 mouse
Enucleate & isolate retina
Transfer to Millicell
insert in 6 well plate

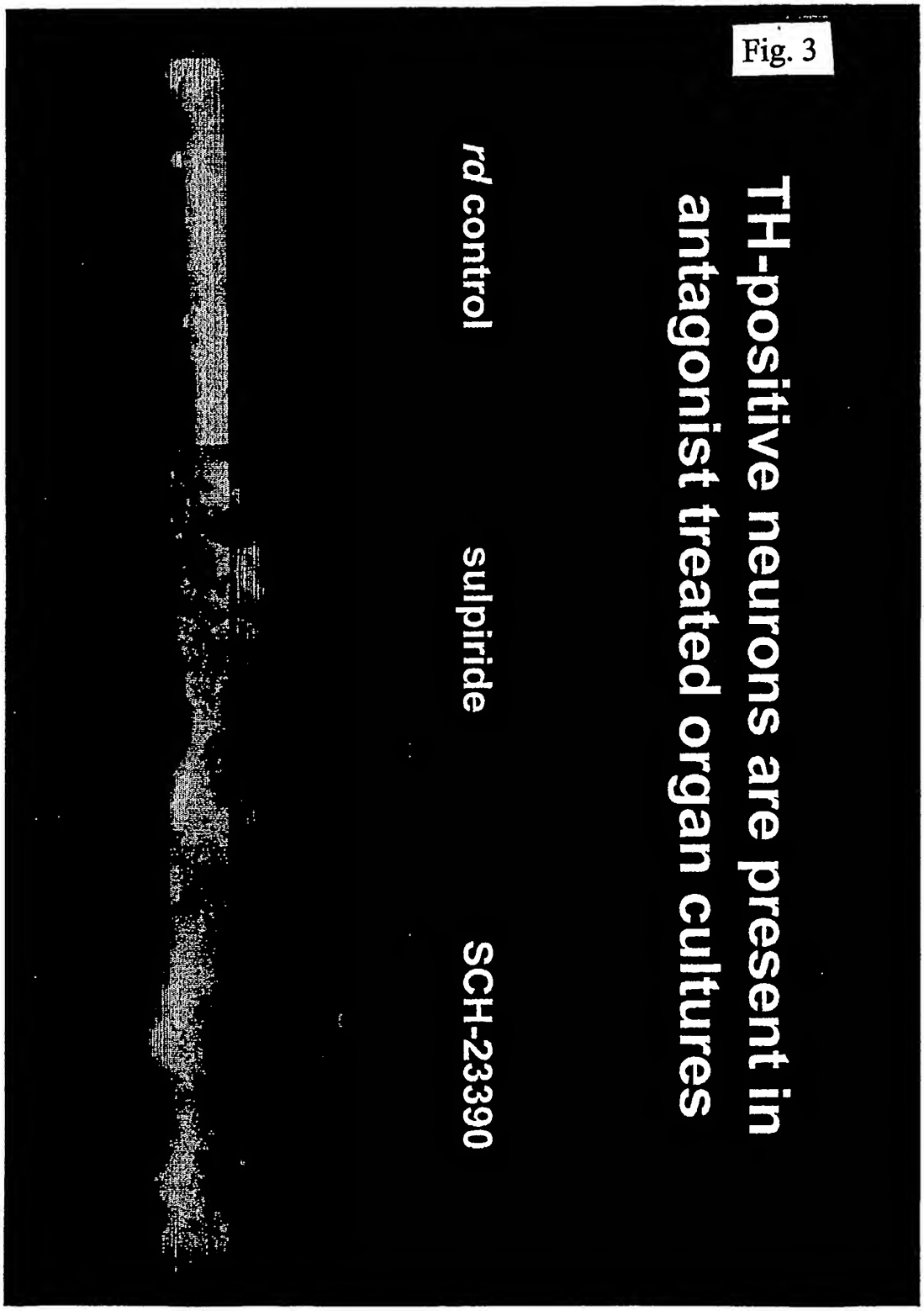


Fig. 3
TH-positive neurons are present in antagonist treated organ cultures
rd control
sulpiride
SCH-23390

Fig. 4

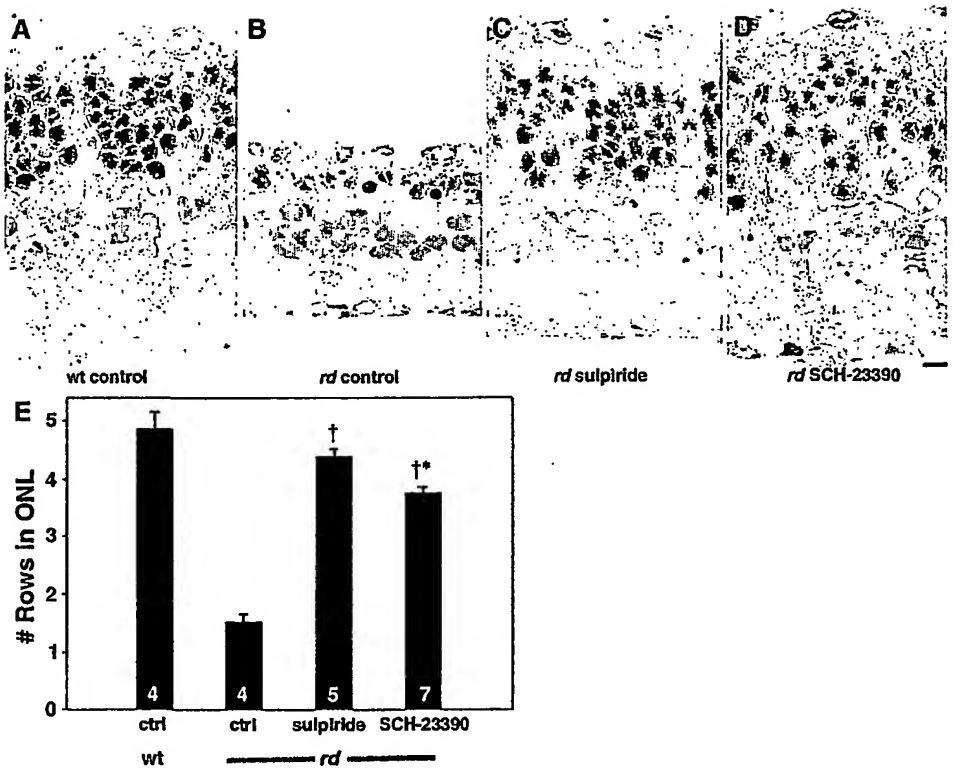

A
B
C
D
wt control
rd control
rd sulpiride
rd SCH-23390
E
Rows in ONL
0
1
2
3
4
5
†
†*
4
4
5
7
ctrl
ctrl
sulpiride
SCH-23390
wt
rd

Fig. 5

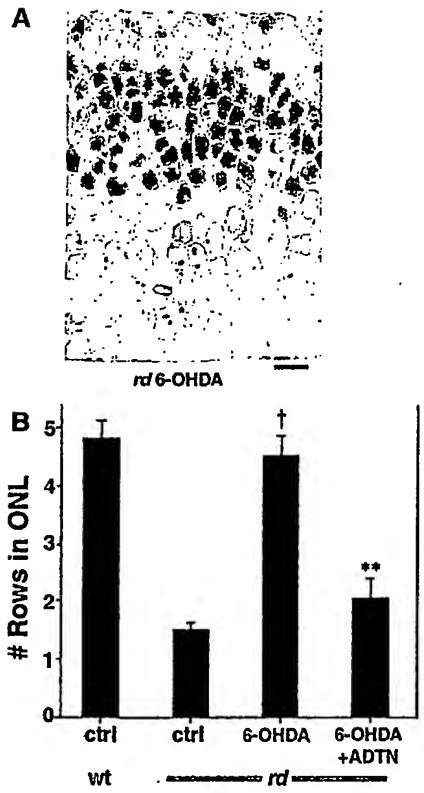
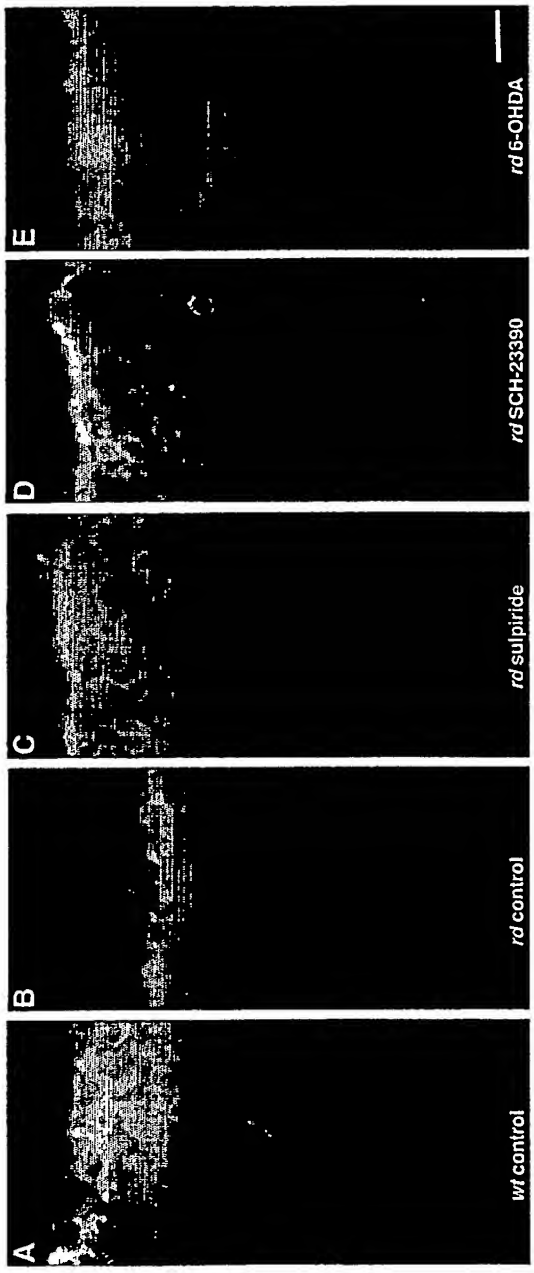

A
rd 6-OHDA
B
Rows in ONL
0
1
2
3
4
5
†
**
ctrl
ctrl
6-OHDA
6-OHDA
+ADTN
wt
rd

Fig. 6


A
wt control
B
rd control
C
rd sulpiride
D
rd SCH-23390
E
rd 6-OHDA